II.1—TRACING BIODEGRADATION WITH STABLE CARBON ISOTOPE MEASUREMENTS OF RESPIRED CO_2

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INTRODUCTION

Laboratory studies were conducted to determine whether stable carbon isotope ratios ($\delta^{13}C$) of respired CO_2 could be used as indicators of substrates being mineralized by microorganisms during biodegradation. Biometer-flask experiments were conducted in which bacteria were grown on pure and complex substrates of varying $\delta^{13}C$ values ranging from -9.8‰ to -29.7‰. These substrates included glucose, acetate, fluoranthene, cordgrass, crude oil, and sedimentary organic matter. Respired CO_2 was collected and measured for $\delta^{13}C$. These measurements demonstrated that CO_2 respired by both an axenic culture and a consortium of microorganisms had $\delta^{13}C$ values close to that of the carbon substrate on which they were grown, especially when the bacteria were actively assimilating the carbon source. For example, when the axenic culture was grown on fluoranthene ($\delta^{13}C$ = -24.4‰), respired CO_2 measured -24.3‰. Similarly, when the consortium was grown on cordgrass ($\delta^{13}C$ = -13.6‰), CO_2 measured -15.7‰. Measurements of CO_2 $\delta^{13}C$ values were used to determine the relative percentages of two carbon sources degraded by bacteria on a mixture of the substrates. Results indicate that $\delta^{13}C$ measurements of CO_2 at polluted field sites might be used to determine whether indigenous microorganisms are biodegrading the constituents of interest (COI) or are consuming nonpollutant organic matter.

There is a challenge in the bioremediation of contaminated field sites to establish that organisms are actually affecting the clean up of the site. This is especially true for in situ bioremediation applications where decreases in COI loading cannot be directly attributed to biodegradation [25]. The effectiveness of bioremediation has been measured by several different methods [2]. Respiration methods, where oxygen loss and/or CO_2 production is measured, are used often. A problem common to this approach is that oxygen is also consumed, and CO_2 is produced during the degradation of indigenous organic compounds. Partial oxidation or incomplete mineralization of the COI poses additional complications, as does chemical oxygen demand. An alternative method is to trace CO_2 production from ^{14}C-labeled model

compounds [2,7,13,16,22]. Although this technique has proven useful for determining the rates of degradation of specific compounds, the behavior of model compounds cannot be extrapolated easily to whole mixtures. Another conventional method is to measure the loss of "parent" compounds directly by gas chromatography (GC) with one of several detectors, such as mass spectrometry (MS) or flame ionization detection (GC/FID). These analytical methods quantify the loss of a "parent" compound but cannot determine if the loss is due to biotic or abiotic processes [10,14,15,19,23,24].

An emerging approach (that takes advantage of the attributes of respirometry and ^{14}C-labeled model compounds while minimizing their weaknesses) is to measure the stable carbon isotope ratio ($\delta^{13}C$) of respired CO_2. For some time now, $\delta^{13}C$ measurements have been used in ecological research to study the flow of organic material through food webs [20]. The strength of this approach derives from the fact that different sources of organic matter have distinct $\delta^{13}C$ values [8]. For example, the majority of terrestrial plants have $\delta^{13}C$ values of -24 to -28‰, most marine higher plants have values between -10 and -16‰, and plankton range from -16 to -22‰. In environments where petroleum $\delta^{13}C$ values (ranging from -26 to -30‰ [21]) do not overlap with that of indigenous organic matter, the mineralization products of these two carbon sources may be discriminated. For example, Aggarwal and Hinchee [1] demonstrated that soil gas CO_2 at contaminated sites differed isotopically from that at uncontaminated sites.

We tested the hypothesis that CO_2 respired by microorganisms has a similar $\delta^{13}C$ value to that of their carbon source. Biometer-flask experiments were conducted with pure and complex substrates of varying $\delta^{13}C$ values (see Table 1). These substrates ranged from glucose, acetate, and fluoranthene (pure compounds) to cordgrass, crude oil, and sedimentary organic matter (complex mixtures). We demonstrate that $\delta^{13}C$ measurements of respired CO_2 are useful tracers of biodegradation in controlled laboratory experiments and discuss the potential for applying this technique to the study of bioremediation at actual field sites.

Table 1 — Structure and Carbon Isotopic Ratio ($\delta^{13}C$) of Substrates

MATERIALS AND METHODS

Biometer Flask Experiments

Biometer flasks (Bellco Biotechnology, Vineland, NJ) consisted of a 250-mL incubation vessel that contained the media, substrates and bacteria, and a sidearm into which was placed NaOH to trap CO_2. Before each experiment, the NaOH was boiled for 2 h to remove trace CO_2 and then stored in gas-tight containers to prevent atmospheric CO_2 contamination. NaOH samples were collected with gas-tight syringes and kept in either Vacutainers® or vials that had been flushed with N_2 gas. Each flask had its own syringe that was acid-washed after each sampling period. Biometer flasks were fitted with two stoppers. One was above the sidearm containing a 15-gauge needle through which NaOH samples could be withdrawn. The other was above the incubation flask containing an ascarite/drierite trap to prevent atmospheric CO_2 contamination when NaOH was withdrawn from the sidearm [3]. Substrates and parameters measured during all experiments are listed in Table 2.

Table 2 — Description of Biometer Flask Experiments

Expt. #	Substrates	Parameters
1	T1: 50 mL M-9 media + 50 mg gluc	$[CO_2]$; bacterial biomass; $\delta^{13}C$ (CO_2, DOC, bacteria)
2	T1: 50 mL M-9 media + 50 mg gluc - NH_4^+; C:N 50:1 T2: 50 mL M-9 media + 50 mg gluc; C:N 13:1 T3: 50 mL M-9 media + 50 mg gluc; C:N 2:1	$[CO_2]$, $\delta^{13}C$ (CO_2)
3	T1: 50 mL M-9 media + 50 mg gluc T2: 50 mL M-9 media + 50 mg gluc; add 50 mg glu after 8 days T3: 50 mL M-9 media + 50 mg gluc; add NH_4^+ after 8 days	$[CO_2]$, $\delta^{13}C$ (CO_2)
4	T1: 50 mL M-9 media + 150 mg acetate	$[CO_2]$, $\delta^{13}C$ (CO_2)
5	T1: 60 mL BH media + 3.5 mg fla T2: killed control (w/ fla)	$[CO_2]$, [fla], δ ^{13}C (CO_2, fla, bacteria)
6	T1: 50 mL M-9 media + 2 g of carbonate-free sediments	$[CO_2]$, $\delta^{13}C$ (CO_2)
7	T1: 50 mL BH media + 6.3 mg fla T2: 50 mL BH media + 14.9 mg gluc T3: 50 mL BH media + 6.3 mg fla + 14.9 mg gluc (1:1 fla to gluc carbon) T4: killed control (w/ fla + gluc)	$[CO_2]$, $\delta^{13}C$ (CO_2)
8	T1: 50 mL M-9 media + 30 mg oil T2: 50 mL M-9 media + 30 mg cordgrass T3: 50 mL M-9 media + 30 mg oil + 30 mg cordgrass T4: killed control (w/ oil and cordgrass)	$[CO_2]$, $\delta^{13}C$ (CO_2)

Abbreviations are as follows: T = treatment; gluc = glucose; fla = fluoranthene; BH = Bushnell-Haas; DOC = dissolved organic carbon

Experiments 1-4, 6, and 8 were conducted with a consortium of bacteria that were originally isolated from Galveston Bay ship channel sediments [12], the composition of which likely changed over the time period of these experiments. The biometer flasks were acid-washed and contained 40-55 mL of media in the growth side and 10 mL of 0.5 N NaOH in the sidearm. All treatments were set up in duplicate. Flasks were kept in a room with a temperature range of 20° to 22° C and were exposed to light during the day. The flasks were shaken at approximately 140 rpm. For Experiment 1, bacteria were isolated for isotopic analysis by filtering the contents in the growth side of the flask through a 4.7-cm Whatman glass fiber/filter (GF/F). The filter was dried in an oven (50° C) and ground with a mortar and pestle.

For the above experiments, M-9 minimal salts media (6.79 g/L Na_2HPO_4, 3.00 g/L KH_2PO_4, 1.00 g/L NH_4Cl, 16.8 g/L NaCl, 0.492 g/L $MgSO_4$, and 0.011 g/L $CaCl_2$) was used. Some treatments in Experiment 4 used different amounts of NH_4Cl to vary C:N. Fifty mL of M-9 media with 50 mg of glucose had a C:N of about 2:1. A C:N of about 13:1 was obtained by reducing NH_4Cl in the media from 1.00 g/L to 0.15 g/L. In turn, a C:N of about 50:1 was obtained by eliminating NH_4Cl from the media and leaving the inoculum as the sole source of nitrogen. The exact C:N could not be obtained because of the uncertainty of the carbon and nitrogen content of the added biomass.

With the exception of Experiment 5, samples were collected at various times over a 4- to 10-day period. The end of each experiment was signaled by a visual observation that the density of the cultures was decreasing. At each sampling, the NaOH containing the absorbed CO_2 was withdrawn from the sidearms of the flasks and replaced with fresh NaOH.

Experiments 5 and 7 were conducted with an axenic culture of *Sphingomonas (Pseudomonas) paucimobilis*, designated EPA505 [18]. EPA505 was initially grown for 2 days on 1.8 g of glucose as the sole carbon source in 500 mL of Bushnell-Haas media (0.2 g/L $MgSO_4$; 0.02 g/L $CaCl_2$; 1.0 g/L KH_2PO_4; 1.0 g/L K_2HPO_4; 1.0 g/L KN_3O; 0.05 g/L $FeCl_3$). The CO_2 respired by the bacteria during this time was trapped in 3 mL of 2N NaOH contained in a test tube in the culture flask to obtain a measurement of the $\delta^{13}C$ value of CO_2 from cells grown on glucose. Fluoranthene was added to the incubation side of the flasks in an acetone solution prior to the addition of media. The acetone was allowed to evaporate, leaving a coating of large fluoranthene crystals on the walls of the flasks. Biometer flasks were filled with media and then sonicated for 30 s to break up the large crystals. Approximately 2-3 mL of 2N NaOH were placed in the sidearms of the flasks to collect CO_2. An aliquot of the EPA505 culture was added to ``live'' biometer flasks to produce a cell concentration of 10^8 CFU/mL. An identical amount of autoclaved cells was added to the remaining killed-cell control flasks. During the time-course experiments, flasks were incubated in

the dark at 30° C (the optimum temperature for growth of EPA505) and shaken at 120 rpm. All treatments were set up in duplicate except in Experiment 7, in which the flasks in treatment 3 (media + fluoranthene + glucose) were set up in triplicate.

Experiment 5 was conducted in a different manner than the rest of the experiments because entire flasks needed to be sacrificed to allow for accurate measurements of fluoranthene concentration and isotopic ratio. At each sampling time, four ``live'' flasks were sacrificed. All four flasks were sampled for CO_2. Fluoranthene was extracted from two of these flasks with methylene chloride and analyzed for concentration and $\delta^{13}C$. The other two flasks were sampled for bacteria. The contents of these flasks were pipetted into fluorinated-ethylene-propylene (FEP) tubes and centrifuged at 10,000 rpm for 10 min. The bacterial pellet was frozen at -70° C for nucleic acid extraction [5]. At 188 h, the four killed-cell control flasks were sampled along with four ``live'' flasks for direct comparison of biotic and abiotic changes in measured parameters.

Analyses of Concentrations

With the exception of Experiments 5 and 7, CO_2 concentrations were estimated from pressure readings on the mass spectrometer that were adjusted for the amount of NaOH used to trap the CO_2. In Experiment 1, bacterial carbon biomass was determined by similarly measuring the quantity of CO_2 produced during combustion for the isotope analyses.

For Experiments 5 and 7, fluoranthene and CO_2 concentrations were determined by gas chromatography. For fluoranthene, the methylene chloride extracts were injected onto a Hewlett-Packard (HP) 5890 Series II/3 gas chromatograph equipped with a 25-m × 0.32-mm HP-5 column and a flame ionization detector (FID). CO_2 concentrations were measured by withdrawing a 25 to 500 μL subsample of the NaOH and injecting it into a crimp-sealed headspace vial containing 250 μL of concentrated phosphoric acid. The liberated CO_2 was analyzed on an HP 5890 Series II/3 gas chromatograph equipped with an automatic headspace analyzer and a 20-m × 0.53-mm Poraplot Q fused-silica column. CO_2 was reduced in the presence of a Ni-catalyst to CH_4, and CH_4 concentrations were measured with an FID.

Stable Carbon Isotope Analyses

Stable carbon isotope ratios are reported in the standard $\delta^{13}C$ notation, as follows:

$$\delta^{13}C_{PDB}(‰) = [(R_{sample}/R_{std}) - 1] \times 1000,$$

where R_{sample} and R_{std} are the $^{13}C/^{12}C$ isotope ratios corresponding to the sample and the conventional Pee Dee Belemnite (PDB) carbonate standard, respectively [6]. By definition, the $\delta^{13}C$ value of PDB is 0.0‰.

The $\delta^{13}C$ values of pure carbon substrates (glucose, acetate, fluoranthene), oil, cordgrass, sediments, bacterial biomass, bacterial nucleic acids, and dissolved organic carbon were measured by using conventional methods [17]. For dissolved organic carbon samples, approximately 15 mL of the filtrate from the bacterial recovery procedure was frozen in a 20 mL vial and lyophilized for isotopic analysis. Similarly, nucleic acid samples, which were dissolved in distilled water, were freeze-dried for 24 h. All samples were placed into precombusted quartz tubes. Copper wire (5 g) and CuO (2.5 g) were added to the samples, and the tubes were evacuated and sealed. In turn, the tubes were heated to 850° C for 2 h to generate CO_2 gas, which was isolated cryogenically on a vacuum line. The carbon isotope content of the CO_2 was measured on a Finnigan 251 carbon/nitrogen/oxygen/sulfer (CNOS) isotope ratio mass spectrometer (precision of ±0.01‰). With glucose as the carbon substrate, a preliminary biometer flask experiment run in triplicate revealed a precision of ±0.8‰ (n = 3) over an 8-day period.

For stable isotope analysis of CO_2 samples collected in Experiments 1-4, 6, and 8, the CO_2 in the NaOH solution was recovered with a modified procedure of Grossman [11]. To a 30-mL flask containing 1 mL of concentrated phosphoric acid and connected to a vacuum line, 7.5 mL of the NaOH solution containing the CO_2 from the biometer experiments were added by injection through a septum. In turn, the CO_2 was cryogenically distilled and stored in a sealed 6-mm o.d. Pyrex tube. The $\delta^{13}C$ value of the CO_2 was measured on the Finnigan 251 CNOS isotope ratio mass spectrometer.

In contrast to the method described above, the $\delta^{13}C$ values of the fluoranthene samples collected by methylene chloride extraction of the biometer flasks and of respired CO_2 (Experiments 5 and 7) were measured using a Finnigan MAT 252 gas-chromatography/combustion/isotope ratio mass spectrometer (GC/C/IRMS). With this system, mixtures of analytes are separated by the GC column. As compounds elute from the GC column, they are swept through a 940° C combustion furnace packed with a mixture of cupric oxide, nickel, and platinum catalysts where carbon constituents are reacted to CO_2 gas. Cogenerated water vapor is removed via a Nafion® elimination trap, and interfering nitrogen oxides are reduced to N_2 gas via a 600° C reduction column. The purified CO_2 flows into the IRMS where its carbon isotopic composition is measured. Reproducibility of $\delta^{13}C$ values for multiple injections of fluoranthene and CO_2 standards by this instrument was better than ±0.2‰. For the determination of the $\delta^{13}C$ values of fluoranthene, 1 μL of the methylene chloride extract was injected into the GC, which was equipped with a 50-m × 0.32-mm i.d. Ultra-2 (Hewlett-Packard) column. For the determination of the $\delta^{13}C$ values of respired

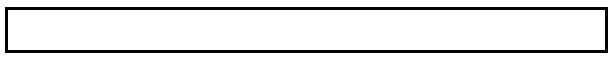
CO_2, an aliquot of the NaOH sample was injected into a headspace vial containing 250 μL of concentrated phosphoric acid. From 50 to 500 μL of the headspace was injected onto the GC/ C/IRMS system. The gas chromatograph was equipped with a 25-m × 0.32-mm Poraplot Q column (Chrompack, Inc.).

RESULTS AND DISCUSSION

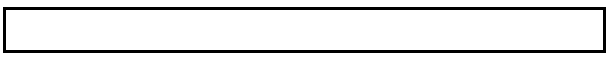
Glucose Mineralization

The ability to distinguish sources of organic matter to microorganisms using stable carbon isotope measurements of respired CO_2 depends on the precision of these analyses. We determined the reproducibility of our $\delta^{13}C$ measurements of CO_2 to be ±0.8‰. This precision was more than sufficient to discriminate among the carbon substrates used in this study ($\delta^{13}C$ = -9.8 to -29.7‰).

Another important consideration is whether the $\delta^{13}C$ of respired CO_2 tracks that of the substrate over various growth stages of the microorganisms. To address this issue, we measured the $\delta^{13}C$ of CO_2, bacterial biomass, and dissolved organic carbon (filtrate) throughout the course of a 10-day experiment (Experiment 1). When $\delta^{13}C$ values of CO_2 fall within ±0.8‰ (the precision of the measurement) of the value of glucose (i.e., -9.0 to -10.6‰), the $\delta^{13}C$ of CO_2 is a good tracer of substrate degradation. Initially during Experiment 1, when cumulative CO_2 production and bacterial biomass increased rapidly (Day 1) (Fig. 1(a)), the $\delta^{13}C$ of CO_2 was within 0.8‰ of the value of glucose (i.e., values fall between the two lines in Fig. 1(b)). After 2 days, however, cumulative CO_2 production decreased, and there was loss of bacterial biomass; concurrently, the $\delta^{13}C$ of CO_2 became increasingly more negative. In contrast, the $\delta^{13}C$ of bacteria and dissolved organic carbon (glucose and bacterial exudates) (Fig. 1(b)) were relatively constant such that their isotopic discrimination from glucose was small throughout the experiment. These results indicate that changes in the $\delta^{13}C$ of CO_2 were influenced by bacterial growth efficiency and not by variations in $\delta^{13}C$ of bacteria or substrate. As long as bacteria were actively assimilating the substrate, the isotopic discrimination between CO_2 and glucose was sufficiently small to make the $\delta^{13}C$ of CO_2 a useful tracer of bacterial substrate.



(a)

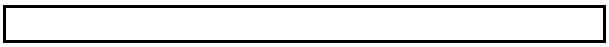


(b)

Fig. 1 — (a) Cumulative CO_2 production (μmol) and the amount of carbon (μmol) in bacterial biomass with glucose as
substrate and (b) the $\delta^{13}C$ of the evolved CO_2, bacterial biomass, and filtrate (glucose and bacterial exudates)

In the above experiment, we determined that increasingly negative $\delta^{13}C$ values of CO_2 occurred when cumulative CO_2 production leveled off. The $\delta^{13}C$ of CO_2 at the beginning and end of many experiments conducted with glucose as substrate was often between -15 and -22‰, which is much more negative than the substrate value (-9.8‰). A different carbon source, possibly one that was added with the inoculum, could have explained the relatively negative $\delta^{13}C$ values of CO_2 at the initiation of the experiments. The inoculum was measured to have a $\delta^{13}C$ of -25.2‰. However, it comprised less than 2% of the amount of carbon added to the media. Thus it was more likely that availability of substrate or nutrients influenced the $\delta^{13}C$ of CO_2, producing large isotopic fractionations.

In order to determine whether the availability of carbon substrate and/or nutrients (ammonium) influenced the $\delta^{13}C$ of CO_2 during the glucose experiments, we investigated the relationship between nitrogen and carbon availability and isotopic discrimination between CO_2 and glucose. In Experiment 2, we provided varying carbon (glucose) to nitrogen (ammonium) atomic ratios (C:N). Production and $\delta^{13}C$ of CO_2 were compared with C:N of 50:1, 13:1, and 2:1 in the media. In flasks with a C:N of 50:1, production of CO_2 was smallest, and the most CO_2 was produced in the flasks with a C:N of 2:1 (Fig. 2(a)). Although the $\delta^{13}C$ of CO_2 (Fig. 2(b)) in all the flasks was relatively negative at the initiation of the experiment, it measured within the precision of the measurement by Day 2. The major difference between the flasks was the amount of time the $\delta^{13}C$ of CO_2 stayed close to the $\delta^{13}C$ value of glucose. In the 2:1 flasks, the $\delta^{13}C$ of CO_2 stayed within 0.8‰ of the value of glucose through Day 6. The isotopic discrimination between CO_2 and glucose increased more quickly in the 13:1 and 50:1 flasks, reaching values outside the precision of the isotope measurement between Days 3 and 6 in the 13:1 flasks and between Days 2 and 3 in the 50:1 flasks.



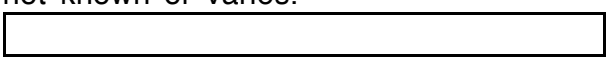
(a)

(b)

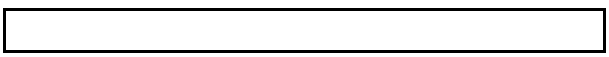
Fig. 2 — (a) Cumulative CO_2 production (μmol) and (b) the $\delta^{13}C$ of evolved CO_2 with glucose as substrate and varying levels of C:N

We hypothesized that increases in isotopic discrimination between CO_2 and carbon substrate are influenced more by substrate availability than nitrogen availability. To test this hypothesis, flasks were set up with glucose as the carbon substrate and a C:N ratio of 2:1. When cumulative CO_2 production leveled off (Day 8), additional glucose was added to one set of flasks, additional nitrogen was added to another set, and the control was unamended (Experiment 3). The addition of carbon resulted in a large increase in CO_2 production whereas the addition of nitrogen did not (Fig. 3(a)). With the addition of carbon, the $\delta^{13}C$ of CO_2 returned to a value within 0.8‰ of that of glucose (Fig. 3(b)). However, the flasks with the added nitrogen and the control flasks continued to experience more and more negative $\delta^{13}C$ values of CO_2.

It is important to emphasize that $\delta^{13}C$ of CO_2 became fairly negative only after cumulative CO_2 production leveled off (i.e., after the majority of CO_2 had already been respired). Thus, if a weighted-average $\delta^{13}C$ of CO_2 is calculated for the final sampling of the experiments discussed above, values ranging from -11.0 to -11.9‰ are obtained. Although these values are more negative than those of the glucose, the isotopic discrimination is fairly small, and $\delta^{13}C$ values of CO_2 should still be good indicators of the substrate being utilized by bacteria even when the growth stage is not known or varies.



(a)



(b)

Fig. 3 — (a) Cumulative CO_2 production (μmol) and (b) the $\delta^{13}C$ of evolved CO_2 with glucose as substrate. After 8 days, more glucose was added to one set, more nitrogen (ammonium) was added to another set, and the control set was unamended

Single Substrates

Having demonstrated that the stable isotope ratio of respired CO_2 can be used as a tracer of glucose degradation by bacteria, we measured the $\delta^{13}C$ of CO_2 respired when individual substrates of different composition and complexity were degraded (Table 1). As examples, we describe the behavior of acetate and fluoranthene. The acetate experiment (Experiment 4) was unique because this molecule contains carbons of distinct isotopic composition [9]. In turn, the fluoranthene experiment (Experiment 5) differed from the others in that an axenic bacteria strain, proven to degrade high-molecular-weight polycyclic aromatic hydrocarbons (PAHs), was used. Earlier attempts to grow the Galveston ship channel consortium on PAHs were not successful; thus we resorted to using a known PAH degrader [18].

With acetate as the substrate, the CO_2 released from Days 4 to 6 had a $\delta^{13}C$ of -28.1‰ (Fig. 4(b)). Galimov [9] reports a difference of 22‰ between carboxyl and methyl carbons of acetate. Based on this difference and the composite $\delta^{13}C$ value of acetate in the media (-18.7‰), we estimated that values for methyl and carboxyl carbons were -7.7‰ and -29.7‰, respectively. Thus, the CO_2 produced early in this experiment was similar to that calculated for the carboxyl carbon. In turn, there was continued production of measurable amounts of CO_2 through Day 10 when the isotope value of respired CO_2 was more similar to that of the methyl carbon (Fig. 4(b)). These results indicate that the bacteria metabolize the acetate carbons sequentially, beginning with the carboxyl carbon and then moving to the methyl carbon.

In the experiment, using fluoranthene as the sole carbon source, fluoranthene concentrations decreased over time, and CO_2 concentrations increased, indicating that fluoranthene was being mineralized (Fig. 5(a)). Initially, 1066 μmol C as fluoranthene

(13.5 mg) were added to the flasks. At t = 0, the measured amount of fluoranthene averaged 1007 μmol C per flask, indicating a recovery efficiency of 94.5%. By Day 2, the concentration of fluoranthene was reduced to 674 μmol C per flask, and it continued decreasing until a final value of 250 μmol C per flask was reached at 188 h. Killed-cell control flasks had an average fluoranthene concentration of 766 μmol C per flask after 188 h, indicating that approximately 29% abiotic loss occurred.

Under active growth conditions, CO_2 concentrations increased from below detection levels to 591 μmol C per flask. Percent mineralization values were calculated based on an initial fluoranthene concentration of 1066 μmol C per flask. After 188 h, 55.5% mineralization of fluoranthene to CO_2 occurred in the ``live'' flasks, whereas only 6.3% mineralization occurred in the "control" flasks.

(a)

(b)

Fig. 4 — (a) Cumulative CO_2 production (μmol) and (b) the $\delta^{13}C$ of evolved CO_2 with acetate as substrate

(a)

(b)

Fig. 5 — (a) Concentration of fluoranthene and cumulative CO_2 production (μmol C) and (b) the $\delta^{13}C$ of fluoranthene, bacterial nucleic acids, and evolved CO_2

The measured $\delta^{13}C$ values for fluoranthene, bacterial nucleic acids, and respired CO_2 in the "live" flasks are shown in Fig. 5(b). The $\delta^{13}C$ value of fluoranthene remained constant with time, averaging -24.5 ± 0.2‰ (n = 8). This value, determined with the GC/C/IRMS system, agrees well with the value of -24.4‰, which was measured for fluoranthene using more conventional techniques [24]. A value of -24.6 ± 0.1‰ (n = 2) was measured for fluoranthene isolated from the killed-cell control flasks. Because the $\delta^{13}C$ value of fluoranthene remained constant over the course of the experiment even though its concentration decreased, it appears that no biotic or abiotic processes resulted in isotopic fractionation of fluoranthene.

Bacterial nucleic acids initially had a $\delta^{13}C$ value of -13.6‰, which is slightly more negative than the value of glucose ($\delta^{13}C$ = -9.8‰). (Glucose was the carbon substrate used to grow the initial cell culture.) After 2 days of incubation on fluoranthene, bacterial nucleic acids reached and maintained a $\delta^{13}C$ value close to that of fluoranthene, averaging -22.7 ± 0.3‰ (n = 6). This value is 1.8‰ more positive than that of fluoranthene. Other studies have also indicated that bacterial nucleic acids tend to be 1 to 2‰ enriched in ^{13}C relative to their carbon source [4,5].

Similar to earlier experiments, the $\delta^{13}C$ value of CO_2 was found to agree closely to the value of the carbon substrate which it was assimilating. When EPA505 was initially incubated on glucose, the $\delta^{13}C$ value of CO_2 measured -10.8‰ (indicated by the line in Fig. 5(b)). Within 2 days of incubation on fluoranthene, the $\delta^{13}C$ value of respired CO_2 averaged -24.3 ± 0.6‰ (n = 4). A value of -23.0 ± 1.0‰ (n = 4) was measured for CO_2 collected from killed-cell control flasks at 188 h. This indicates that the CO_2 produced in these flasks resulted from the abiotic degradation of fluoranthene or that there was a small living population of bacteria in the killed-cell flasks.

In order to evaluate the utility of $\delta^{13}C$ values of CO_2 as indicators of biodegradation of more complex substrates, we conducted similar studies to the experiments discussed above using sedimentary organic matter (Experiment 6), oil (Experiment 8; treatment 1), and cordgrass (Experiment 8; treatment 2) as carbon substrates. In all cases, the $\delta^{13}C$ of CO_2 was similar to that of the substrate as long as the bacteria were respiring actively (Fig. 6). The results of all single substrate experiments are summarized in Fig. 6, where the $\delta^{13}C$ of CO_2 is plotted relative to the $\delta^{13}C$ of the carbon substrate. As discussed earlier, methyl and carboxyl isotope values of acetate were estimated. Therefore, the $\delta^{13}C$ of CO_2 may be closer to the real value than it appears in these cases. We conclude that stable isotope measurements of CO_2 produced during degradation can be performed with sufficient precision to distinguish between substrates.

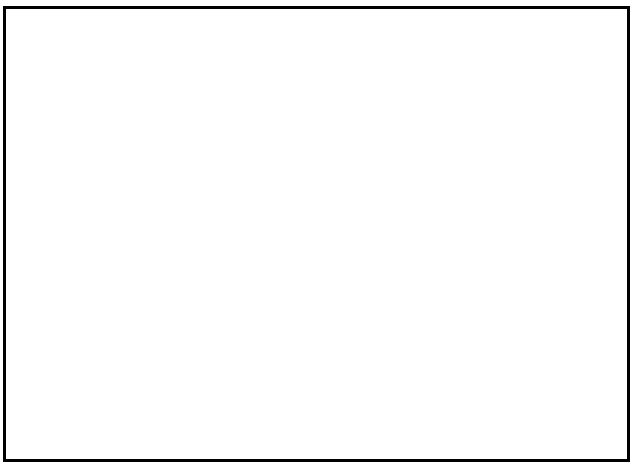
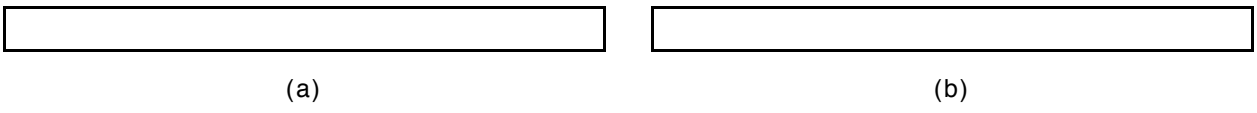


Fig. 6 — The $\delta^{13}C$ of evolved CO_2 at peak CO_2 production vs the $\delta^{13}C$ of the substrate on which the bacteria were grown. Values for the methyl and carboxyl carbons of acetate were calculated from Galimov [9].

Multiple Substrates

In order to see if $\delta^{13}C$ measurements of CO_2 could be used to determine the carbon source used by bacteria when a mixture of substrates was available, a relatively simple experiment was conducted in which EPA505 was offered either fluoranthene, glucose, or a 1:1 mixture of fluoranthene and glucose carbon (Experiment 7). Cumulative CO_2 production plots (Fig. 7(a)) indicate that bacteria in all flasks were actively growing. Therefore, the $\delta^{13}C$ values of CO_2 from the different flasks should be strong indicators of the carbon substrates being used. By the last sampling, 71% of the substrate had been mineralized to CO_2 in the fluoranthene-only flasks (495 μmol C initially added as fluoranthene), 65% of the substrate was mineralized in the glucose-only flasks (495 μmol C initially added as glucose), and 52% had been mineralized in the fluoranthene and glucose flasks (990 μmol C initially added as a 1:1 mixture of fluoranthene and glucose). Only 23% of the mixture of fluoranthene and glucose was mineralized in the killed-cell control flasks.

The $\delta^{13}C$ values of CO_2 from the various flasks are illustrated in Fig. 7(b). Initially, both the fluoranthene-only and glucose-only flasks gave off CO_2 with fairly negative $\delta^{13}C$ values relative to the



(a) (b)

Fig. 7 — (a) Cumulative CO_2 production (μmol) and (b) the $\delta^{13}C$ of evolved CO_2 with glucose and fluoranthene combinations

substrate values. The CO_2 of the fluoranthene-only flasks measured -26.9‰ at the first sampling. This is 2.5‰ more negative than the value of fluoranthene. Similarly, CO_2 of the glucose-only flasks measured -21.7‰, which is 11.9‰ more negative than the value of glucose. However, by Day 1, the $\delta^{13}C$ values of CO_2 from both the fluoranthene-only and glucose-only flasks agreed much more closely to the substrate values, measuring -23.6‰ for the fluoranthene-only flasks and -14.0‰ for the glucose-only flasks. They agreed even more closely to the substrate values by the end of the experiment, measuring -24.0‰ and -12.4‰, respectively.

The $\delta^{13}C$ values of CO_2 from the fluoranthene/glucose flasks measured approximately halfway between the $\delta^{13}C$ values of CO_2 from the fluoranthene-only and glucose-only flasks throughout the duration of the experiment. This indicates that both fluoranthene and glucose were being used as carbon substrates by the bacteria, with the bacteria showing no preferential mineralization of either compound. By comparing the average $\delta^{13}C$ value of CO_2 from the fluoranthene/glucose flasks at each time point with the respective fluoranthene-only and glucose-only flasks, we calculated the percentage of CO_2 that was contributed by the mineralization of fluoranthene relative to that from the mineralization of glucose. At the initial sampling, 55.3% of the CO_2 resulted from the mineralization of fluoranthene. Similarly, 57.6% of the CO_2 came from the mineralization of fluoranthene at Day 1, 39.3% at Day 2, 38.2% at Day 3, and 43.3% at Day 4. In comparison, although less than half the amount of CO_2 was produced in the killed-cell control flasks relative to the live fluoranthene/glucose flasks, the $\delta^{13}C$ value of the CO_2 in the killed flasks was similar the that in the live flasks at the end of the experiment. Whereas 43.3% of the CO_2 produced in the live flasks was from the mineralization of fluoranthene, 45.3% was from fluoranthene in the killed flasks. These results indicate that there was a population of cells that survived the autoclaving and went on to degrade the mixture of glucose and fluoranthene as observed in the live flasks.

In Experiment 8, either oil, cordgrass, or a mixture of the two complex carbon sources was offered to the bacterial consortium in order to see if the $\delta^{13}C$ of respired CO_2 could be used to determine the proportions of each substrate mineralized by the microorganisms (Fig. 8). The $\delta^{13}C$ of CO_2 in the oil flasks approached the value of oil (-29.7‰), but after 2 days, there was an enrichment in ^{13}C of the CO_2. The CO_2 in the cordgrass flasks also approached the value of cordgrass (-13.6‰) and then leveled off at -14.5‰. The $\delta^{13}C$ of CO_2 from the oil/cordgrass flask was intermediate between those values of the oil and cordgrass alone at the beginning and then approached the value of the CO_2 from the oil flask. The $\delta^{13}C$ values of CO_2 in the killed control flasks were close to those of the cordgrass flasks, indicating that abiotic degradation of cordgrass was occurring.

(a)

(b)

Fig. 8 — (a) Cumulative CO_2 production (μmol) and (b) the $\delta^{13}C$ of evolved CO_2 with cordgrass and oil combinations

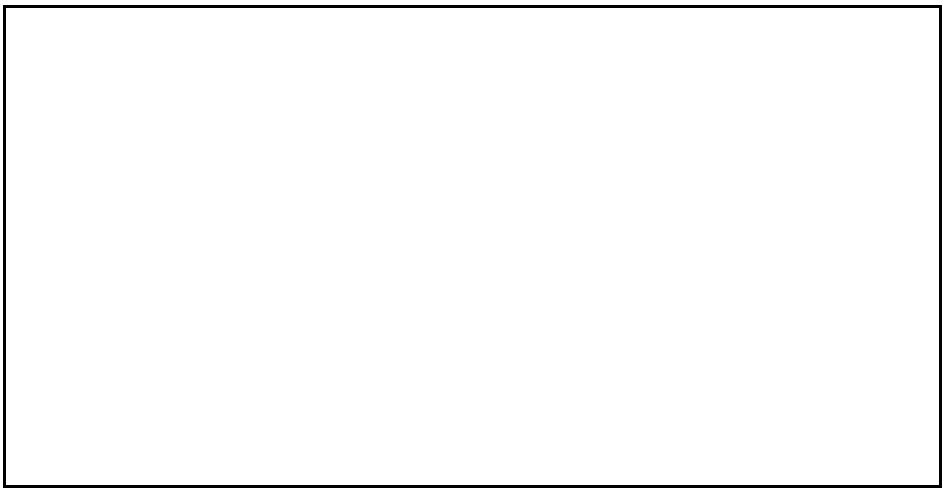


Fig. 9 — Conceptual diagram of how the $\delta^{13}C$ of CO_2 could be used to determine the source of organic matter being degraded during a bioremediation procedure

We used the $\delta^{13}C$ of CO_2 to estimate relative percentages of the substrates utilized by the bacteria in the oil/cordgrass flasks. After correction for the blank (killed control), the weighted-average $\delta^{13}C$ value of CO_2 in the oil-only flasks measured -17.3‰ by the last sampling. The cordgrass-only flasks measured -27.8‰, and the oil/cordgrass flasks measured -24.2‰. Based on isotopic balance of these means, about 70% of the evolved CO_2 resulted from degradation of oil in the oil/cordgrass flasks.

CONCLUSIONS

Results of these biometer-flask studies indicate that $\delta^{13}C$ measurements can be used to trace the degradation and mineralization of organic matter by microorganisms. Extrapolating these results to field studies will require a slightly different approach, however. In a contaminated environment, it will be necessary to measure the isotopic ratio of remineralized CO_2 in both a contaminated and an uncontaminated control site. These sites must be of similar soil and vegetation composition. Measurements can first be made using biometer-flask experiments. If the isotopic ratios between contaminated and control sites differ, then measurements can be performed in the field, as depicted in Fig. 9. If significant degradation of the COI are occurring, we hypothesize that the isotopic ratios of the soils in the control and contaminated sites will differ as observed in the laboratory study. In turn, when degradation has ceased (i.e., low nutrients, site is clean, only nonbioavailable residual degradation products of the COI remain), then the control and contaminated sites will be characterized by CO_2 with similar $\delta^{13}C$ values.

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